REMARKS

In the Office Action mailed March 29, 2005, Claims 1-15, 21 and 23-35 have been rejected under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement and written description in the specification for biological membranes which are not intact cells or liposomes. The Examiner has alleged that the specification does not provide any description of any biological membrane other than the reference on page 11 that the membrane may be "any membranous or lipid-containing material obtained from biological systems such as cells, tissues, bacteria, viruses, or components thereof." Further, the Examiner has alleged that the specification does not provide examples or guidance as to how to prepare membranous or lipid-containing material from the listed sources such that the material is capable of incorporating a chelator lipid and encapsulating an active material, or for the therapeutic administration thereof. The Examiner has cited Forssen et al. (1998) Adv. Drug Deliv. Rev., 29:249-271 ("Forssen et al.") as evidence of "the art-recognized unpredictability in the targeted delivery of liposomes and other vehicles to particular cells."

Applicants respectfully submit that the present specification provides sufficient written description and an enabling disclosure for the subject matter of Claims 1-15, 21 and 23-35.

To satisfy the written description requirement, the specification must describe the invention in sufficient detail such that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention. There is a strong presumption that an adequate written description of the claimed invention is present when the application is filed. In re Wertheim, 541 F.2d 257, 263, 191 U.S.P.Q. 90, 97 (C.C.P.A. 1976). Further, information which

is well known in the art need not be described in detail in the specification. Hybritech, Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1379-80, 231 U.S.P.Q. 81, 90 (Fed. Cir. 1986).

Biological membranes are sufficiently described in the present specification, for example at page 11, lines 29-30. The description therein is sufficient to satisfy the requirements of 35 U.S.C. § 112, first paragraph, since at the filing date of the present invention, biological membranes were well known in the art.

Well before the priority date of the present application, the scientific literature was replete with publications, including monographs, describing biological membranes. As an example thereof, submitted herewith is a copy of Chapter 10 of the standard text in the relevant area, Biochemistry, Second Edition, 1981, Stryer, ed. This chapter, entitled "Introduction to Biological Membranes," demonstrates that in 1981, knowledge of biological membranes was abundant. The Examiner's attention is directed to page 216, which describes the reconstitution of functional membranes. The list of selected readings at page 230 provides further evidence that biological membranes were well known before the filing date of the present application. For example, the Methods of Enzymology, volume 32, which is subtitled "Biomembranes Part A," was published in 1974, and even the last monograph in this series was published in 1991.

Accordingly, at the filing date of the present application the level of skill and knowledge in the art with respect to biological membranes was extremely high. The specificity of the present disclosure is thus sufficient to meet the written description requirement.

Further, the specification provides a disclosure that enables one of skill in the art to make and use the claimed invention.

One of ordinary skill in the art, with the teachings of the specification, can make and use biological membranes into which have been incorporated amphiphilic molecules modified by covalent attachment of a metal chelating group. The specification provides detailed teaching for making amphiphilic molecules having an attached chelating group, for example at page 27, and detailed teaching for incorporating such molecules into membranes, for example at page 18. As the Examiner has noted, the specification provides specific guidance for biological membranes that are intact cells and liposomes. The specification is also replete with guidance for incorporating the chelator lipids into all biological membranes. See, e.g. page 17, line 27 – page 20, line 16. As discussed hereinabove, such biological membranes and methods of using them were very well known to those of skill in the art.

Thus with the teachings of the specification and the abundant knowledge in the art of biological membranes, one of skill in the art could practice the claimed invention in the absence of undue experimentation. The specification need not provide an example if the invention is disclosed in a manner such that it can be practiced without undue experimentation.

The Examiner has further alleged that the specification fails to provide an enabling disclosure for the therapeutic administration of biological membranes, or for targeting cells using such membranes.

The specification discloses such methods, and provides a prophetic example, as the Examiner has noted. This disclosure is sufficient to satisfy the enablement requirement, and must be presumed accurate in the absence of a reason to doubt the statements in the specification. In re Marzocchi, 439 F.2d 220, 224, 169 U.S.P.Q. 367, 370 (C.C.P.A. 1971).

The Examiner has not provided any reason to doubt the presumptive accuracy of the specification. Forssen et al. has been cited by the Examiner as concluding that "while advances in lipid based drug delivery systems is encouraging, substantial hurdles remain to be overcome...." Rather than providing any reason to doubt the accuracy of the present specification, Forssen et al. in fact support the present disclosure. Forssen et al. disclose that vesicle-formulated drugs have received regulatory approval and achieved commercial success, and are able to deliver a few percent of an administered dose of a therapeutic agent to a target. Forssen et al. do not teach that this art is unpredictable. Rather, the reference teaches that liposomal drug delivery is therapeutically successful, and further improvements could lead to more clinical advantages. Forssen at p. 251.

Accordingly, one of ordinary skill in the art can practice the claimed invention in the absence of undue experimentation. Forssen et al. provide no evidence to the contrary, and in fact support the teachings of the specification.

In view of the foregoing comments, withdrawal of the rejections of Claims 1-15, 21 and 23-35 under 35 U.S.C. § 112, first paragraph, is respectfully requested.

Claims 1-15 and 30-35 have been rejected under 35 U.S.C. § 112, second paragraph as allegedly indefinite. Claim 1 allegedly lacks proper antecedent basis for the terms "the chelator lipid" and "the membranous structure" of step (i) of Claim 4 and the "metal chelating residues" of step (ii) of Claim 4. Claims 1 and 4 have been amended to provide appropriate antecedent basis.

Further, the claims are allegedly confusing in the use of the terms "receptor domain," "targeting molecule," "targetable molecules" and "receptor domain and/or other targeting

molecule." Claims 1, 2, 4, 5, 21, 27, 30 and 33 have been amended to replace the allegedly confusing terms with the terms "molecule to be engrafted" and "engrafted molecule." Engrafted molecules are disclosed in the specification, for example at page 16, lines 19-21, and include receptors, ligands, molecules having binding partners on target cells or tissues, such as VEGF, and molecules capable of modifying an immunological response, such as co-stimulatory molecules. Further, for clarification it is noted that Applicants do not adopt the various definitions set forth in the Office Action at pages 10-11.

Claims 3 and 9 are allegedly indefinite for failing to further limit Claim 1. Claims 3 and 9 have been cancelled without prejudice.

Claims 30-35 are allegedly indefinite in the use of the words "anchoring or engrafting" since the specification states that the terms are interchangeable. The term "anchoring or" has been deleted from Claim 30.

Claim 35 is allegedly further indefinite in the use of the term "when." Claim 35 has been amended to delete this term and to depend upon Claim 31.

In view of the foregoing comments and amendments, withdrawal of the rejection under 35 U.S.C. § 112, second paragraph, is respectfully requested.

Claims 2-15, 22-26, 28, 29 and 32-35 have been objected to for improperly starting with the article "a." The claims have been amended to begin with a definite article. Withdrawal of the objection is respectfully requested.

Claim 6 has been cancelled without prejudice. Claims 21, 27, 28 and 30 have been amended for clarity and consistency.

In view of the foregoing comments and amendments, it is respectfully submitted that the present application is in condition for allowance. Favorable consideration and allowance of all pending claims is earnestly solicited.

Respectfully submitted,

DORSEY & WHITNEY LLP

Date: September 29, 2005

By: _____

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CHAPTER 10

INTRODUCTION TO BIOLOGICAL MEMBRANES

We now turn to biological membranes, which are organized assemblies consisting mainly of proteins and lipids. The functions carried out by membranes are indispensable for life. Membranes give cells their individuality by separating them from their environment. *Membranes are highly selective permeability barriers* rather than impervious walls because they contain specific molecular *pumps* and *gates*. These transport systems regulate the molecular and ionic composition of the intracellular medium. Eucaryotic cells also contain internal membranes that form the boundaries of organelles such as mitochondria, chloroplasts, and lysosomes. Functional specialization in the course of evolution has been closely linked to the formation of these compartments.

Membranes also control the flow of information between cells and their environment. They contain *specific receptors for external stimuli*. The movement of bacteria toward food, the response of target cells to hormones such as insulin, and the perception of light are examples of processes in which the primary event is the detection of a signal by a specific receptor in a membrane. In turn, *some membranes generate signals,* which can be chemical or electrical. Thus, membranes play a central role in biological communication.

The two most important *energy conversion processes* in biological systems are carried out by membrane systems that contain highly ordered arrays of enzymes and other proteins. *Photosynthesis,* in which light is converted into chemical-bond energy, occurs in the

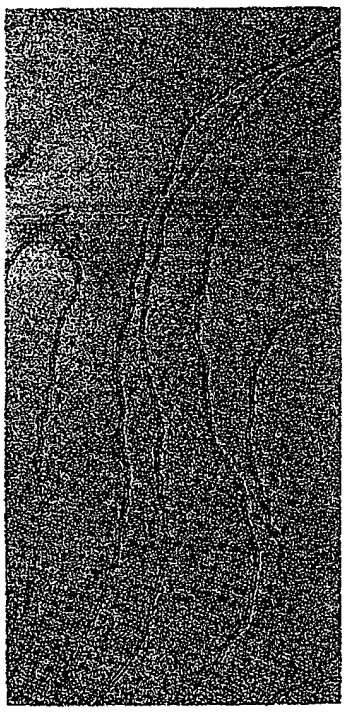


Figure 10-1
Electron micrograph of a preparation of plasma membranes from red blood cells. [Courtesy of Dr. Vincent Marchesi.]

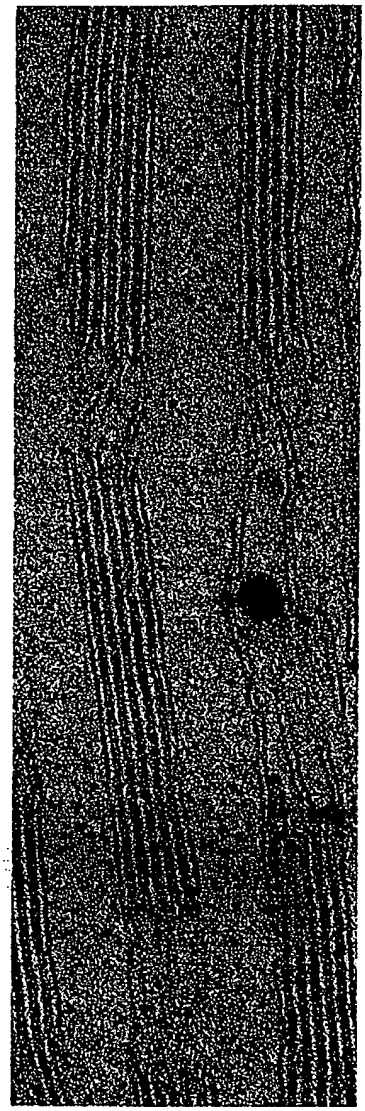


Figure 10-2
Light is converted into chemical-bond energy by photosynthetic assemblies in the thylakoid membranes of chloroplasts. [Courtesy of Dr. Myron Ledbetter. From *Introduction to the Fine Structure of Plant Cells* by M. C. Ledbetter and K. R. Porter (Springer-Verlag, 1970).]

inner membranes of chloroplasts (Figure 10-2), whereas *oxidative phosphorylation*, in which adenosine triphosphate (ATP) is formed by the oxidation of fuel molecules, takes place in the inner membranes of mitochondria. These and other membrane processes will be discussed in detail in later chapters. This chapter deals with some essential features that are common to most biological membranes.

COMMON FEATURES OF BIOLOGICAL MEMBRANES

Membranes are as diverse in structure as they are in function. However, they do have in common a number of important attributes:

1. Membranes are *sheetlike structures*, only a few molecules thick, that form *closed boundaries* between compartments of different composition. The thickness of most membranes is between 60 and 100 Å.

2. Membranes consist mainly of *lipids* and *proteins*. The weight ratio of protein to lipid in most biological membranes ranges from 1:4 to 4:1. Membranes also contain *carbohydrates* that are linked to lipids and proteins.

3. *Membrane lipids are relatively small molecules* that have both a hydrophilic and a hydrophobic moiety. These lipids spontaneously form *closed bimolecular sheets* in aqueous media. These *lipid bilayers* are barriers to the flow of polar molecules.

4. *Specific proteins mediate distinctive functions of membranes.* Proteins serve as pumps, gates, receptors, energy transducers, and enzymes. Membrane proteins are intercalated into lipid bilayers, which create a suitable environment for the action of these proteins.

5. Membranes are *noncovalent assemblies*. The constituent protein and lipid molecules are held together by many noncovalent interactions, which are cooperative in character.

6. Membranes are *asymmetric*. The inside and outside faces of membranes are different.

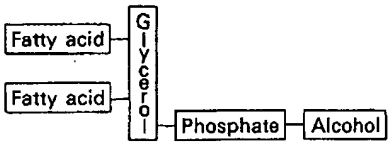
7. Membranes are *fluid structures*. Lipid molecules diffuse rapidly in the plane of the membrane, as do proteins, unless anchored by specific interactions. Membranes can be regarded as *two-dimensional solutions of oriented proteins and lipids.*

PHOSPHOLIPIDS ARE THE MAJOR CLASS OF MEMBRANE LIPIDS

Lipids are a group of biomolecules that are strikingly different from amino acids and proteins. By definition, lipids are water-insoluble biomolecules that have high solubility in organic solvents such as

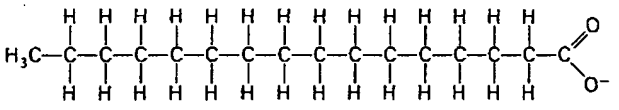
chloroform. Lipids have a variety of biological roles: they serve as fuel molecules, as highly concentrated energy stores, and as components of membranes. The first two roles of lipids will be discussed in Chapter 18. Here, the concern is with lipids as membrane constituents. The three major kinds of membrane lipids are *phospholipids, glycolipids,* and *cholesterol.*

Let us start with phospholipids, because they are abundant in all biological membranes. Phospholipids are derived from either *glycerol,* a three-carbon alcohol, or *sphingosine,* a more complex alcohol. Phospholipids derived from glycerol are called *phosphoglycerides.* A phosphoglyceride consists of a glycerol backbone, two fatty acid chains, and a phosphorylated alcohol.

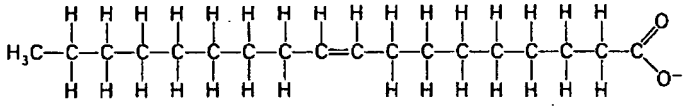


Components of a phosphoglyceride

The *fatty acid chains* in phospholipids and glycolipids usually contain an even number of carbon atoms, typically between 14 and 24. The 16- and 18-carbon fatty acids are the most common ones. In animals, the hydrocarbon chain in fatty acids is unbranched. Fatty acids may be saturated or unsaturated. The configuration of double bonds in unsaturated fatty acids is nearly always *cis.* As will be discussed shortly, the length and the degree of unsaturation of fatty acid chains in membrane lipids have a profound effect on membrane fluidity. The structures of the ionized form of two common fatty acids—palmitic acid (C_{16}, saturated) and oleic acid (C_{18}, one double bond)—are shown below. They will be referred to as palmitate and oleate to emphasize the fact that they are ionized under physiological conditions. The nomenclature of fatty acids is discussed in Chapter 18.



Palmitate
(Ionized form of palmitic acid)



Oleate
(Ionized form of oleic acid)

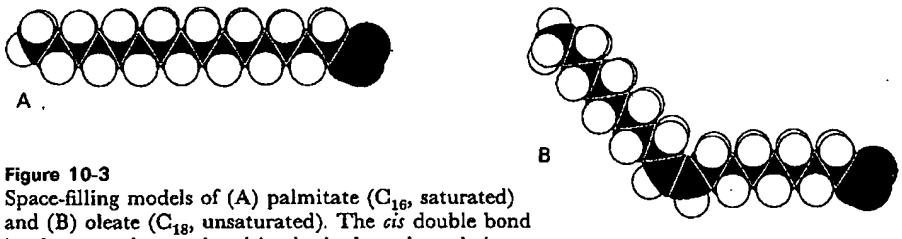


Figure 10-3
Space-filling models of (A) palmitate (C_{16}, saturated) and (B) oleate (C_{18}, unsaturated). The *cis* double bond in oleate produces a bend in the hydrocarbon chain.

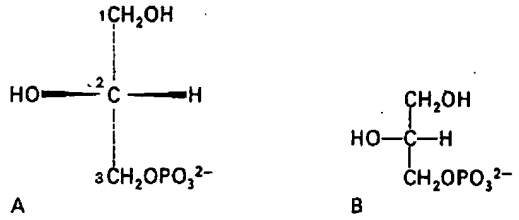
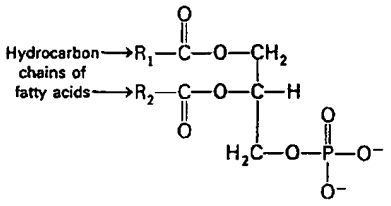


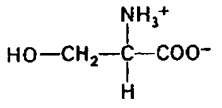
Figure 10-4
Absolute configuration of the glycerol 3-phosphate moiety of membrane lipids: (A) H and OH, attached to C-2, are in front of the plane of the page, whereas C-1 and C-3 are behind it; (B) Fischer representation of this structure. In a Fischer projection, horizontal bonds denote bonds in front, whereas vertical bonds denote bonds behind the plane of the page.



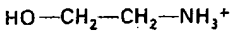
Phosphatidate
(Diacylglycerol 3-phosphate)

In phosphoglycerides, the hydroxyl groups at C-1 and C-2 of glycerol are esterified to the carboxyl groups of two fatty acid chains. The C-3 hydroxyl group of the glycerol backbone is esterified to phosphoric acid. The resulting compound, called *phosphatidate* (or diacylglycerol-3-phosphate), is the simplest phosphoglyceride. Only small amounts of phosphatidate are present in membranes. However, it is a key intermediate in the biosynthesis of the other phosphoglycerides.

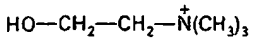
The major phosphoglycerides are derivatives of phosphatidate. The phosphate group of phosphatidate becomes esterified to the hydroxyl group of one of several alcohols. The common alcohol moieties of phosphoglycerides are serine, ethanolamine, choline, glycerol, and inositol.



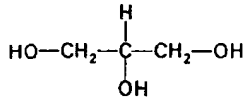
Serine



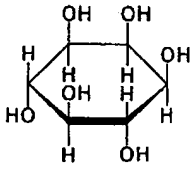
Ethanolamine



Choline

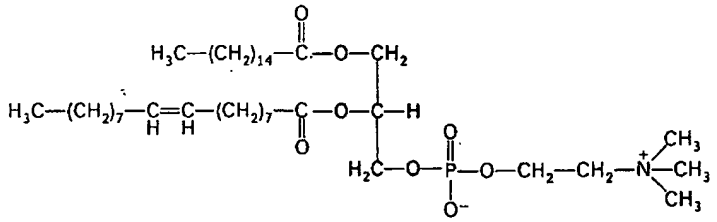


Glycerol



Inositol

Now let us link some of these components to form phosphatidyl choline, a phosphoglyceride found in most membranes of higher organisms.



A phosphatidyl choline
(1-Palmitoyl-2-oleoyl-phosphatidyl choline)

The structural formulas of the other principal phosphoglycerides—namely, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, and diphosphatidyl glycerol—are given in Figure 10-5.

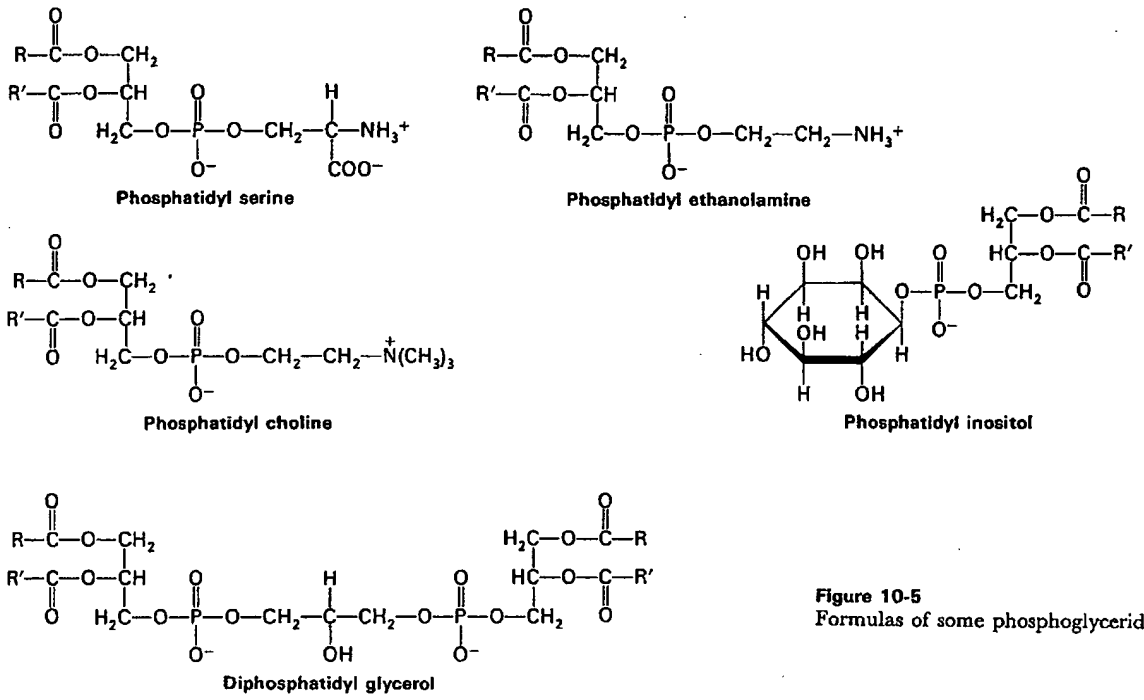
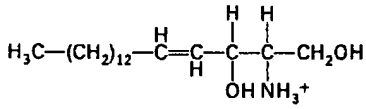
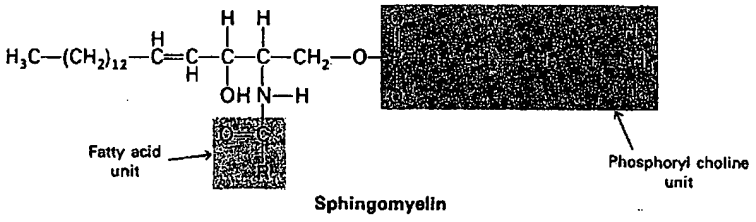


Figure 10-5
Formulas of some phosphoglycerides.

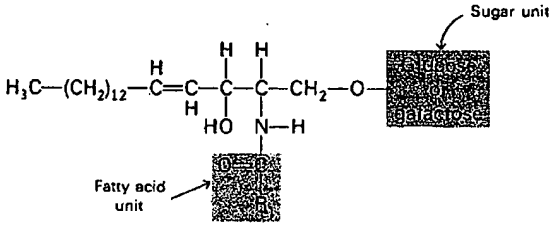
Sphingomyelin is the only phospholipid in membranes that is not derived from glycerol. Instead, the backbone in sphingomyelin is *sphingosine,* an amino alcohol that contains a long, unsaturated hydrocarbon chain. In sphingomyelin, the amino group of the sphingosine backbone is linked to a fatty acid by an amide bond. In addition, the primary hydroxyl group of sphingosine is esterified to phosphoryl choline. As will be shown shortly, the conformation of sphingomyelin resembles that of phosphatidyl choline.





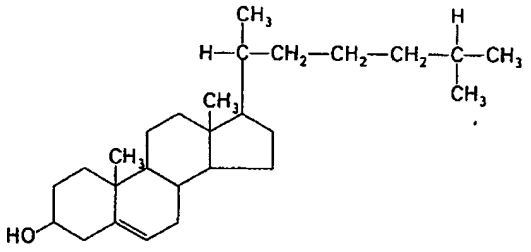
MANY MEMBRANES ALSO CONTAIN GLYCOLIPIDS AND CHOLESTEROL

Glycolipids, as their name implies, are *sugar-containing lipids.* In animal cells, glycolipids, like sphingomyelin, are derived from sphingosine. The amino group of the sphingosine backbone is acylated by a fatty acid, as in sphingomyelin. Glycolipids differ from sphingomyelin in the nature of the unit that is linked to the primary hydroxyl group of the sphingosine backbone. In glycolipids, one or more sugars (rather than phosphoryl choline) are attached to this group. The simplest glycolipid is *cerebroside,* in which there is only one sugar residue, either glucose or galactose. More complex glycolipids, such as *gangliosides,* contain a branched chain of as many as seven sugar residues.



Cerebroside
(A glycolipid)

Another important lipid in some membranes is *cholesterol.* This sterol is present in eucaryotes but not in most procaryotes. The plasma membranes of eucaryotic cells are usually rich in cholesterol, whereas the membranes of their organelles typically have lesser amounts of this neutral lipid.



Cholesterol

PHOSPHOLIPIDS AND GLYCOLIPIDS READILY FORM BILAYERS

The repertoire of membrane lipids is extensive, perhaps even bewildering at first sight. However, they possess a critical common structural theme: *membrane lipids are amphipathic molecules.* They contain both a *hydrophilic* and a *hydrophobic* moiety (Table 10-1).

Table 10-1
Hydrophobic and hydrophilic units of membrane lipids

Membrane lipid	*Hydrophobic unit*	*Hydrophilic unit*
Phosphoglycerides	Fatty acid chains	Phosphorylated alcohol
Sphingomyelin	Fatty acid chain and hydrocarbon chain of sphingosine	Phosphoryl choline
Glycolipid	Fatty acid chain and hydrocarbon chain of sphingosine	One or more sugar residues
Cholesterol	Entire molecule except for OH group	OH group at C-3

Let us look at a space-filling model of a phosphoglyceride, such as phosphatidyl choline (Figure 10-6). Its overall shape is roughly rectangular. The two fatty acid chains are approximately parallel to one another, whereas the phosphoryl choline moiety points in the opposite direction. Sphingomyelin has a similar conformation (Figure 10-7). The sugar group of a glycolipid occupies nearly the same

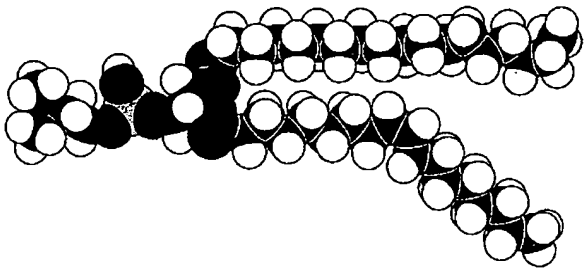


Figure 10-6
Space-filling model of a phosphatidyl choline molecule.

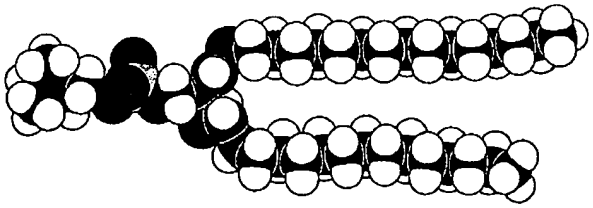


Figure 10-7
Space-filling model of a sphingomyelin molecule.

position as the phosphoryl choline unit of sphingomyelin. Therefore, the following shorthand has been adopted to represent these membrane lipids. The hydrophilic unit, also called the *polar head group,* is represented by a circle, whereas the hydrocarbon tails are depicted by straight or wavy lines (Figure 10-8).

Now let us consider the arrangement of phospholipids and glycolipids in an aqueous medium. It is evident that their polar head groups will have affinity for water, whereas their hydrocarbon tails will avoid water. This can be accomplished by forming a *micelle,* in

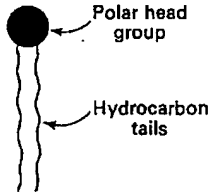


Figure 10-8
Symbol for a phospholipid or glycolipid molecule.

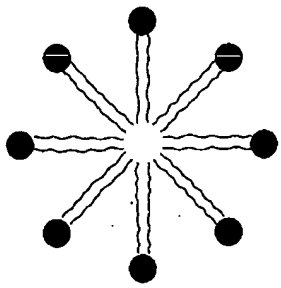


Figure 10-9
Diagram of a section of a micelle formed from phospholipid molecules.

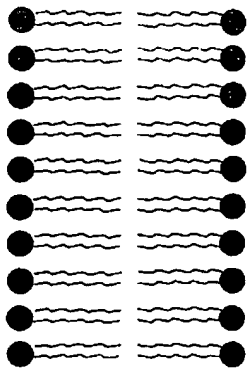


Figure 10-10
Diagram of a section of a bilayer membrane formed from phospholipid molecules.

which the polar head groups are on the surface and the hydrocarbon tails are sequestered inside (Figure 10-9).

Another arrangement that satisfies both the hydrophilic and hydrophobic preferences of these membrane lipids is a *bimolecular sheet,* which is also called a *lipid bilayer* (Figure 10-10). *In fact, the favored structure for most phospholipids and glycolipids in aqueous media is a bimolecular sheet rather than a micelle.* The preference for a bilayer structure is of critical biological importance. A micelle is a limited structure, usually less than 200 Å in diameter. In contrast, a bimolecular sheet can have macroscopic dimensions, such as a millimeter (10^7 Å). Phospholipids and glycolipids are key membrane constituents because they readily form extensive bimolecular sheets. Furthermore, these sheets serve as permeability barriers, yet they are quite fluid.

The formation of lipid bilayers is a *self-assembly process.* In other words, the structure of a bimolecular sheet is inherent in the structure of the constituent lipid molecules, specifically in their amphipathic character. The formation of lipid bilayers from glycolipids and phospholipids is a rapid and spontaneous process in water. *Hydrophobic interactions are the major driving force for the formation of lipid bilayers.* Recall that hydrophobic interactions also play a dominant role in the folding of proteins in aqueous solution. Water molecules are released from the hydrocarbon tails of membrane lipids as these tails become sequestered in the nonpolar interior of the bilayer. This release of water results in a large increase in entropy. Furthermore, there are *van der Waals attractive forces* between the hydrocarbon tails. These van der Waals forces favor close packing of the hydrocarbon tails. Finally, there are favorable *electrostatic* and *hydrogen-bonding* interactions between the polar head groups and water molecules. Thus, lipid bilayers are stabilized by the full array of forces that mediate molecular interactions in biological systems.

LIPID BILAYERS ARE NONCOVALENT, COOPERATIVE STRUCTURES

Another important feature of lipid bilayers is that they are *cooperative structures.* They are held together by many *reinforcing, noncovalent interactions.* Phospholipids and glycolipids cluster together in water to minimize the number of exposed hydrocarbon chains. A pertinent analogy is the clustering together of sheep in the cold to minimize the area of exposed body surface. Clustering is also favored by the van der Waals attractive forces between adjacent hydrocarbon chains. These energetic factors have three significant biological consequences: (1) lipid bilayers have an inherent tendency to be *extensive;* (2) lipid bilayers will tend to *close on themselves* so that there are no ends with exposed hydrocarbon chains, which results in the formation of a compartment; and (3) lipid bilayers are *self-sealing* because a hole in a bilayer is energetically unfavorable.

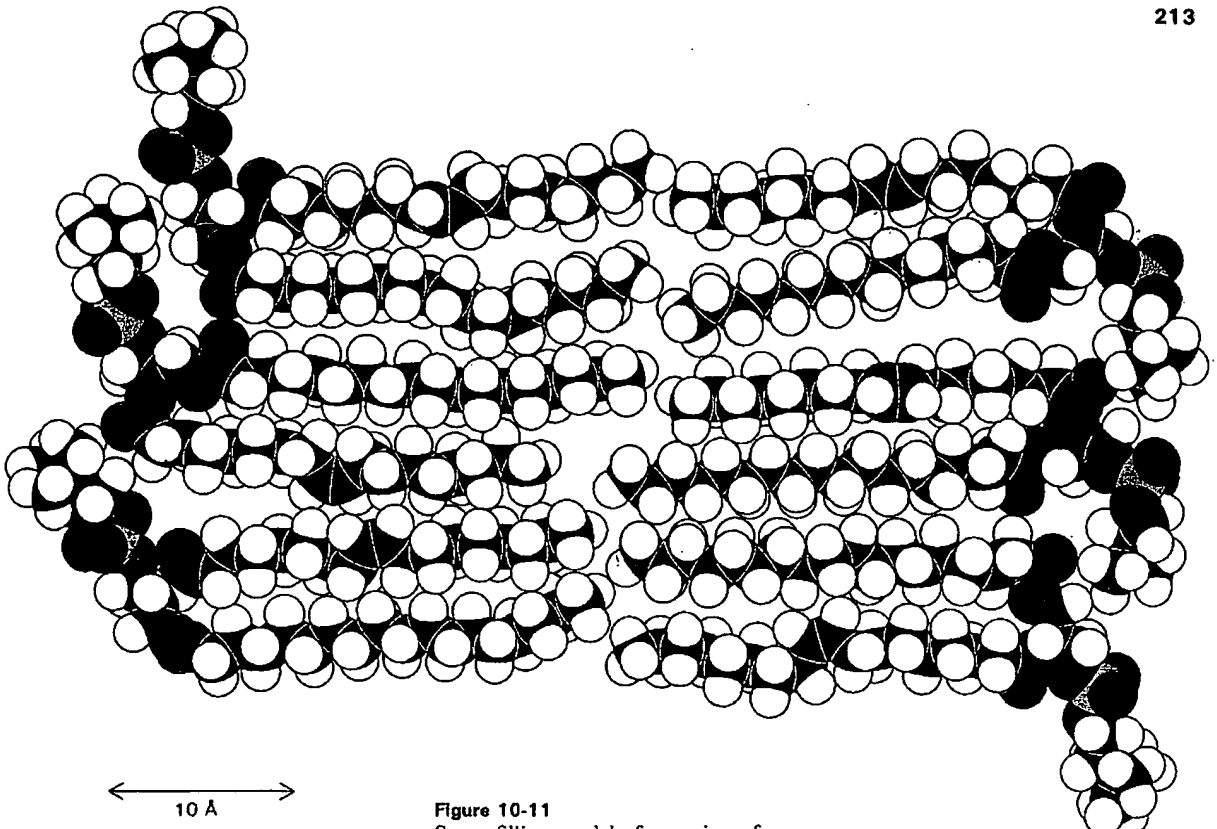


Figure 10-11
Space-filling model of a section of a highly fluid phospholipid bilayer membrane.

LIPID BILAYERS ARE HIGHLY IMPERMEABLE TO IONS AND MOST POLAR MOLECULES

The permeabilities of lipid bilayers have been measured in two well-defined *synthetic systems: lipid vesicles* and *planar bilayer membranes.* These model systems have been sources of insight into a major function of biological membranes—namely, their role as permeability barriers. The key finding is that lipid bilayers are inherently impermeable to ions and most polar molecules.

Lipid vesicles (also known as *liposomes*) are aqueous compartments enclosed by a lipid bilayer (Figure 10-12). They can be formed by suspending a suitable lipid, such as phosphatidyl choline, in an aqueous medium. This mixture is then *sonicated* (i.e., agitated by high-frequency sound waves) to give a dispersion of closed vesicles that are quite uniform in size. Alternatively, vesicles can be prepared by rapidly mixing a solution of lipid in ethanol with water.

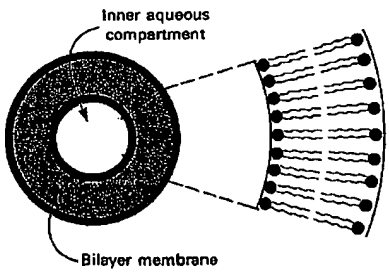


Figure 10-12
Diagram of a lipid vesicle.

This can be accomplished by injecting the lipid through a fine needle. Vesicles formed by these methods are nearly spherical in shape and have a diameter of about 500 Å. Larger vesicles (of the order of 10^4 Å, or 1 μm, in diameter) can be prepared by slowly evaporating the organic solvent from a suspension of phospholipid in a mixed solvent system.

Ions or molecules can be trapped in the aqueous compartment of lipid vesicles by forming them in the presence of these substances (Figure 10-13). For example, if vesicles 500 Å in diameter are

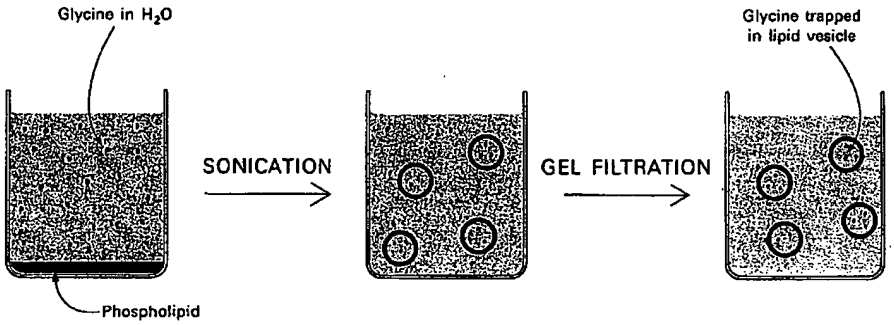


Figure 10-13
Preparation of a suspension of lipid vesicles containing glycine molecules.

formed in a 0.1 M glycine solution, about two thousand molecules of glycine will be trapped in each inner aqueous compartment. These glycine-containing vesicles can be separated from the surrounding solution of glycine by dialysis or by gel-filtration chromatography. The permeability of the bilayer membrane to glycine can then be determined by measuring the rate of efflux of glycine from the inner compartment of the vesicle to the ambient solution. These lipid vesicles are valuable not only for permeability studies. They fuse with the plasma membrane of many kinds of cells and can thus be used to introduce a wide variety of impermeable substances into cells. The selective fusion of lipid vesicles with particular kinds of cells is a promising means of controlling the delivery of drugs to target cells.

The other well-defined synthetic membrane is a *planar bilayer membrane*. This structure can be formed across a 1-mm hole in a partition between two aqueous compartments. Such a membrane is very well suited for electrical studies because of its large size and simple geometry. Paul Mueller and Donald Rudin showed that a large bilayer membrane can be readily formed in the following way. A fine paint brush is dipped into a membrane-forming solution, such as phosphatidyl choline in decane. The tip of the brush is then stroked across a hole (1 mm in diameter) in a partition between two aqueous media. The lipid film across the hole thins spontaneously; the excess lipid forms a torus at the edge of the hole. A planar bilayer membrane consisting primarily of phosphatidyl choline is formed within a few minutes. The electrical conduction properties of this macroscopic bilayer membrane can be readily

studied by inserting electrodes into each aqueous compartment (Figure 10-14). For example, its permeability to ions is determined by measuring the current across the membrane as a function of the applied voltage.

Permeability studies of lipid vesicles and electrical conductance measurements of planar bilayers have shown that *lipid bilayer membranes have a very low permeability for ions and most polar molecules.* Water is a conspicuous exception to this generalization; it readily traverses such membranes. The range of measured permeability coefficients is very wide (Figure 10-15). For example, Na^+ and K^+ traverse these membranes 10^9 times more slowly than does H_2O. Tryptophan, a zwitterion at pH 7, crosses the membrane 10^3 times more slowly than indole, a structurally related molecule that lacks ionic groups. *The permeability coefficients of small molecules are correlated with their solubility in a nonpolar solvent relative to their solubility in water.* This relationship suggests that a small molecule might traverse a lipid bilayer membrane in the following way: first, it sheds its solvation shell of water; then, it becomes dissolved in the hydrocarbon core of the membrane; finally, it diffuses through this core to the other side of the membrane, where it becomes resolvated by water.

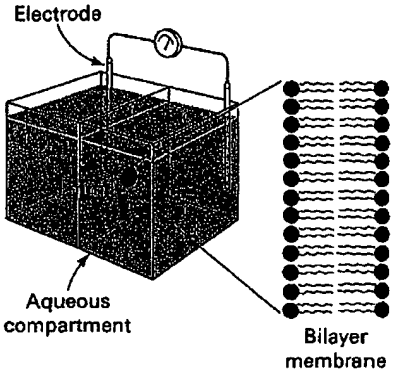


Figure 10-14
Experimental arrangement for the study of planar bilayer membranes. A bilayer membrane is formed across a 1-mm hole in a septum that separates two aqueous compartments.

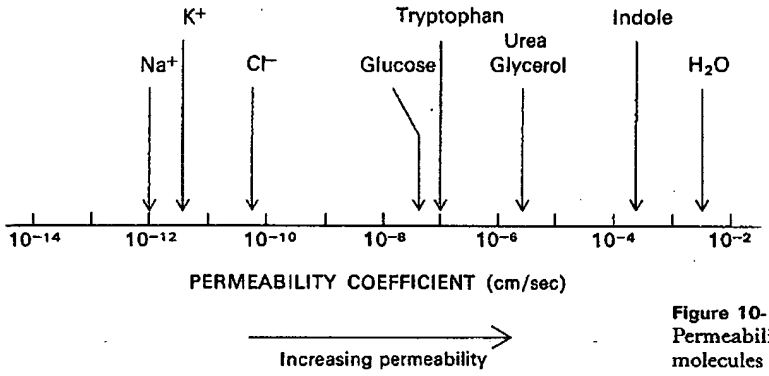


Figure 10-15
Permeability coefficients of some ions and molecules in lipid bilayer membranes.

PROTEINS CARRY OUT MOST MEMBRANE PROCESSES

We now turn to membrane proteins, which are responsible for most of the dynamic processes carried out by membranes. Membrane lipids form a permeability barrier and thereby establish compartments, whereas *specific proteins mediate distinctive membrane functions,* such as transport, communication, and energy transduction. Membrane lipids create the appropriate environment for the action of such proteins.

Membranes differ in their protein content. Myelin, a membrane that serves as an insulator around certain nerve fibers, has a low

content of protein (18%). Lipid, the major molecular species in myelin, is well suited for insulation. In contrast, the plasma membranes of most other cells are much more active. They contain many pumps, gates, receptors, and enzymes. The protein content of these plasma membranes is typically 50%. Membranes involved in energy transduction, such as the internal membranes of mitochondria and chloroplasts, have the highest content of protein, typically 75%.

The major proteins in a membrane can be readily visualized by *SDS–polyacrylamide-gel electrophoresis*. In this technique, the membrane to be analyzed is first solubilized in a 1% solution of sodium dodecyl sulfate (SDS). This detergent disrupts most protein-protein and protein-lipid interactions. The solution is layered on top of an acrylamide gel containing SDS, which is then subjected to an electric field for a few hours. The electrophoretic mobility of many proteins in this gel depends on their mass rather than on their net charge in the absence of SDS. The negative charge contributed by SDS molecules bound to the protein is much larger than the net charge of the protein itself. A pattern of bands appears when the gel is stained with a dye such as coomassie blue. A few micrograms of a protein can be visualized in this way. The gel electrophoresis patterns of three membranes—the plasma membrane of erythrocytes, the photoreceptor membrane of retinal rod cells, and the sarcoplasmic reticulum membrane of muscle—are shown in Figure 10-16. The gel patterns reveal that these three membranes have very different protein compositions. Furthermore, they differ in the number of kinds of polypeptide chains and in distribution of mass. In short, *membranes that perform different functions have different proteins.*

$H_3C—(CH_2)_{10}—CH_2OSO_3^-\ Na^+$

Sodium dodecyl sulfate (SDS)

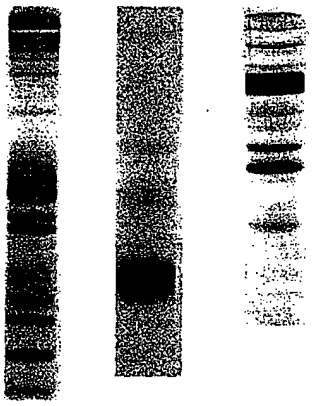


Figure 10-16
SDS–polyacrylamide-gel patterns of (A) the plasma membrane of erythrocytes, (B) the disc membranes of retinal rod cells, and (C) the sarcoplasmic reticulum membrane of muscle cells. [Courtesy of Dr. Theodore Steck (part A) and Dr. David MacLennan (part C).]

FUNCTIONAL MEMBRANE SYSTEMS CAN BE RECONSTITUTED FROM PURIFIED COMPONENTS

Numerous membrane proteins have been solubilized and purified (see Chapters 34 to 37). Some of them are active in detergent solution. For example, rhodopsin, a photoreceptor protein, has the same 500-nm absorption band in detergent solution as it does in the retinal disc membrane. Furthermore, the prosthetic group of this protein undergoes the same structural change on illumination in both environments. Calsequestrin, a calcium-binding protein from the sarcoplasmic reticulum of muscle, retains this ion-binding property when it is removed from its membrane environment. The calcium pump (called the Ca^{2+} ATPase) from this membrane system has also been isolated. In fact, functionally active vesicles can be formed from a mixture of phospholipid and the pump protein. These vesicles accumulate Ca^{2+} if ATP, the energy source for the pump, is provided. *The reconstitution of functionally active membrane systems from purified components is a powerful experimental approach in the elucidation of membrane processes.*

SOME MEMBRANE PROTEINS ARE DEEPLY IMBEDDED IN THE LIPID BILAYER

Some membrane proteins can be released by relatively mild means, such as extraction by a high ionic strength solution (e.g., 1 M NaCl). Other membrane proteins are bound much more tenaciously and can be separated only by using a detergent (Figure 10-17) or an

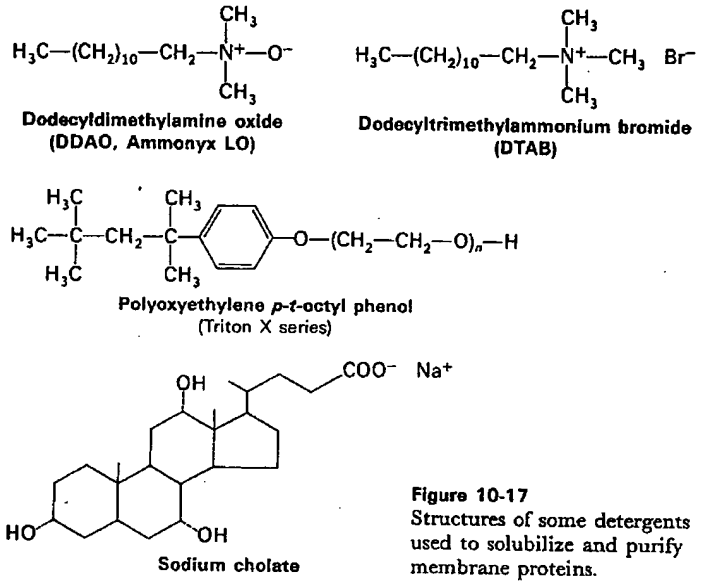


Figure 10-17
Structures of some detergents used to solubilize and purify membrane proteins.

organic solvent. Membrane proteins can be classified as being either *peripheral* or *integral* on the basis of this difference in dissociability (Figure 10-18). Integral proteins interact extensively with the hydrocarbon chains of membrane lipids and so they can be released only by agents that compete for these nonpolar interactions. In contrast, peripheral proteins are bound to membranes by electrostatic and hydrogen-bond interactions and so these polar interactions can be disrupted by adding salts or by changing the pH. It now appears that most peripheral membrane proteins are bound to the surfaces of integral proteins.

Freeze-fracture electron microscopy is a valuable technique for ascertaining whether proteins are located in the interior of biological membranes. Cells or membrane fragments are rapidly frozen at liquid-nitrogen temperatures. The frozen membrane is then fractured by the impact of a microtome knife. Cleavage usually occurs along a plane in the middle of the bilayer (Figure 10-19). Hence, extensive regions *within* the lipid bilayer are exposed. These exposed regions can then be shadowed with carbon and platinum, which yields a replica of the interior of the bilayer. The external surfaces of membranes can also be viewed by combining freeze-fracture and

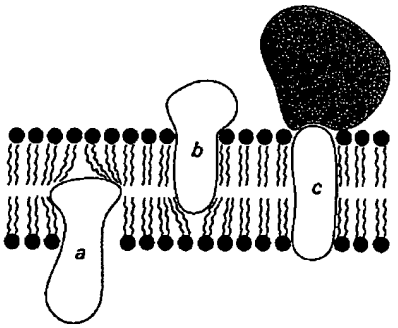


Figure 10-18
Integral membrane proteins (*a*, *b*, *c*) interact extensively with the hydrocarbon region of the bilayer. Peripheral membrane proteins (*d*) bind to the surface of integral membrane proteins.

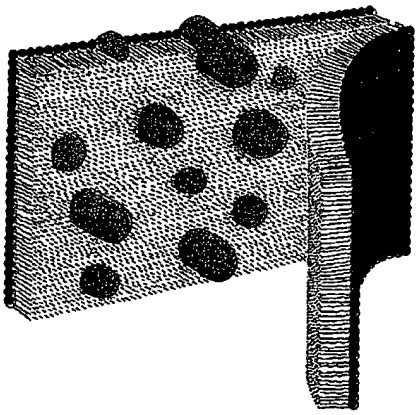


Figure 10-19
Technique of freeze-fracture electron microscopy. The cleavage plane passes through the middle of the bilayer membrane. [After S. J. Singer. *Hosp. Pract.* 8(1973):81.]

deep-etching techniques. First, the interior of a frozen membrane is exposed by fracturing. The ice that covers one of the adjacent membrane surfaces is then sublimed away; this process is termed deep-etching. The combined technique, called *freeze-etching electron microscopy,* provides a view of the interior of a membrane and of both its surfaces. An attractive feature of the freeze-etching technique is that fixatives and dehydrating agents are not required.

Freeze-etching studies have provided direct evidence for the presence of integral proteins in many biological membranes. Erythrocyte membrane, for example, contains a high density of globular particles, approximately 75 Å in diameter, in its interior (Figure 10-20). The inside of the sarcoplasmic reticulum membrane is also

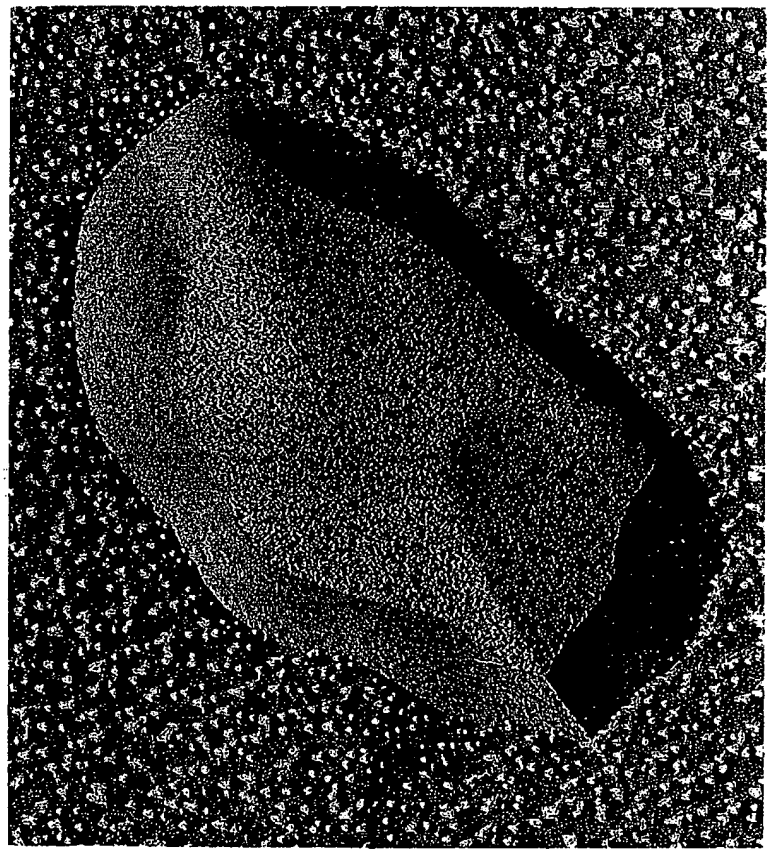


Figure 10-20
Freeze-etch electron micrograph of the plasma membrane of red blood cells. The interior of the membrane, which has been exposed by fracture of the membrane, is rich in globular particles that have a diameter of about 75 Å. These particles are integral membrane proteins. [Courtesy of Dr. Vincent Marchesi.]

rich in globular particles. In contrast, synthetic bilayers formed from phosphatidyl choline yield smooth fracture faces. Also, the fracture faces of large areas of myelin membranes are smooth, as might be expected for a relatively inert membrane that serves primarily as an insulator.

Erythrocytes have been choice objects of inquiry in studies of membranes because of their ready availability and relative simplicity. They lack organelles and thus have only a single membrane, the plasma membrane. Nearly all of the cytoplasmic contents of these cells can be released by osmotic hemolysis to give *ghosts,* which are quite pure plasma membranes. The SDS–polyacrylamide-gel pattern of this membrane preparation is shown in Figure 10-21. More than ten bands are evident when this gel is stained with coomassie blue. The major ones are numbered and referred to as bands 1, 2, 3, 4.1, 4.2, 5, 6, and 7 (Figure 10-21). Staining with the periodic-acid–Schiff (PAS) reagent brings out several proteins that are rich in carbohydrate. These bands are designated as PAS-1, PAS-2, PAS-3, and PAS-4.

What are the locations of these proteins in the red-cell membrane? The proteins corresponding to bands 1, 2, 4.1, 4.2, 5, and 6 can be extracted from the membranes by altering the ionic strength or pH of the medium, and so they are peripheral proteins. Furthermore, they are unaffected when intact red cells or sealed ghosts are incubated with a variety of proteolytic enzymes. In contrast, they are extensively digested when leaky ghosts are treated with proteases. *Hence, it can be concluded that these peripheral proteins are located on the cytoplasmic face of the red-cell membrane.* Band 6 is glyceraldehyde 3-phosphate dehydrogenase, a glycolytic enzyme (p. 263), whereas band 5 is actin, a key protein in muscle contraction and cell motility (p. 816). Bands 1 and 2, called *spectrin,* associate to form an extended filamentous network. Spectrin in concert with other proteins probably *stabilizes and regulates the shape of the red-cell membrane,* which changes markedly in going through small blood vessels (Figure 10-22). Furthermore, red cells are subject to great mechanical stress when pumped by the heart.

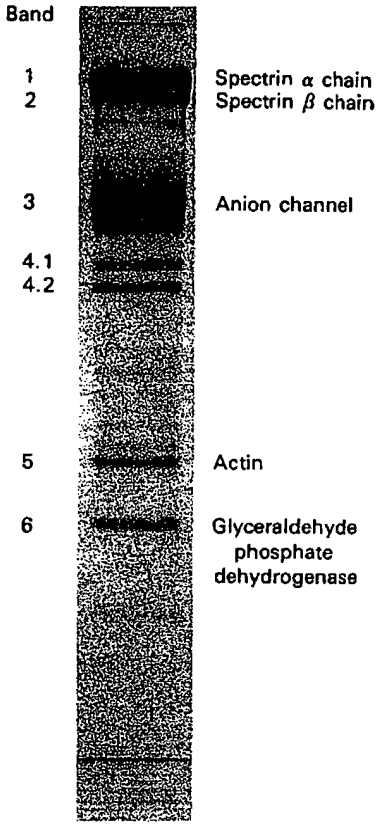


Figure 10-21
SDS-polyacrylamide-gel pattern of the erythrocyte membranes. The gel was stained with coomassie blue. [Courtesy of Dr. Vincent Marchesi.]

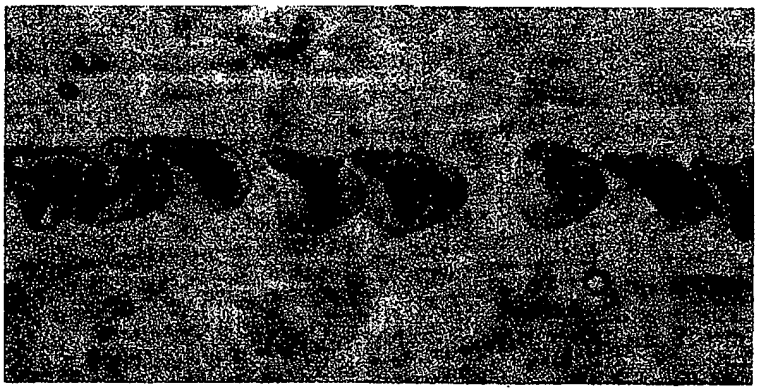


Figure 10-22
Erythrocytes are reversibly deformed as they flow through a small blood vessel. [From P. I. Brånemark. *Intravascular Anatomy of Blood Cells in Man* (Basel: S. Karger AG), 1971.]

In contrast, bands 3 and 7 and all four PAS bands can be dissociated from the red-cell membrane only by detergents or organic solvents. Hence, they are integral membrane proteins. This inference is reinforced by freeze-fracture electron microscopy (Figure 10-20), which shows that some red-cell membrane proteins are deeply embedded in the hydrocarbon region of the membrane.

THE ANION CHANNEL AND GLYCOPHORIN SPAN THE RED-CELL MEMBRANE

The red-cell membrane contains an *anion channel* that makes it permeable to HCO_3^- and Cl^-. The rapid exchange of these ions across the membrane is essential for the transport of CO_2 by red cells. Recent studies have shown that the anion channel is a dimer of the 95-kdal *band-3 protein*, which comprises a quarter of the total protein in a red-cell membrane. The location and orientation of the band-3 protein have been investigated by subjecting intact red cells, leaky ghosts, and inside-out vesicles to proteolysis by chymotrypsin. The cleavage pattern depends on whether chymotrypsin has access to the outer surface, the inner surface, or both surfaces of these membranes. These experiments revealed that *the band-3 protein is located on both sides of the red-cell membrane and that all molecules of this protein point in the same direction* (Figure 10-23). Moreover, its carbohydrate units

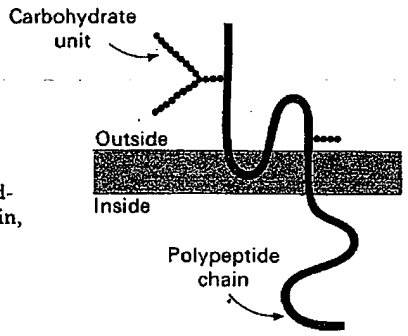


Figure 10-23
Schematic diagram of a model of the band-3 protein (anion channel) in red-cell membranes. [After R. S. Weinstein, J. K. Khodadad, and T. L. Steck. *J. Supramol. Struct.* 8(1978):325.]

are located on the outer surface. The transmembrane nature of the band-3 protein fits nicely with its function as a channel.

The other well-characterized transmembrane protein in red cells is *glycophorin A*, which consists of sixteen oligosaccharide units attached to a single polypeptide chain. Indeed, 60% of the mass of this glycoprotein is carbohydrate, and so its name (derived from the Greek words meaning "to carry sugar") is apt. The abundance of carbohydrate in glycophorin is the reason that it stains intensely with the PAS reagent. In fact, glycophorin corresponds to the *PAS-1 band*. Proteolytic, chemical-modification, and electron-microscopic studies have revealed that glycophorin A consists of three domains: (1) an amino-terminal region containing all of the carbohydrate units, which is located on the outer face of the mem-

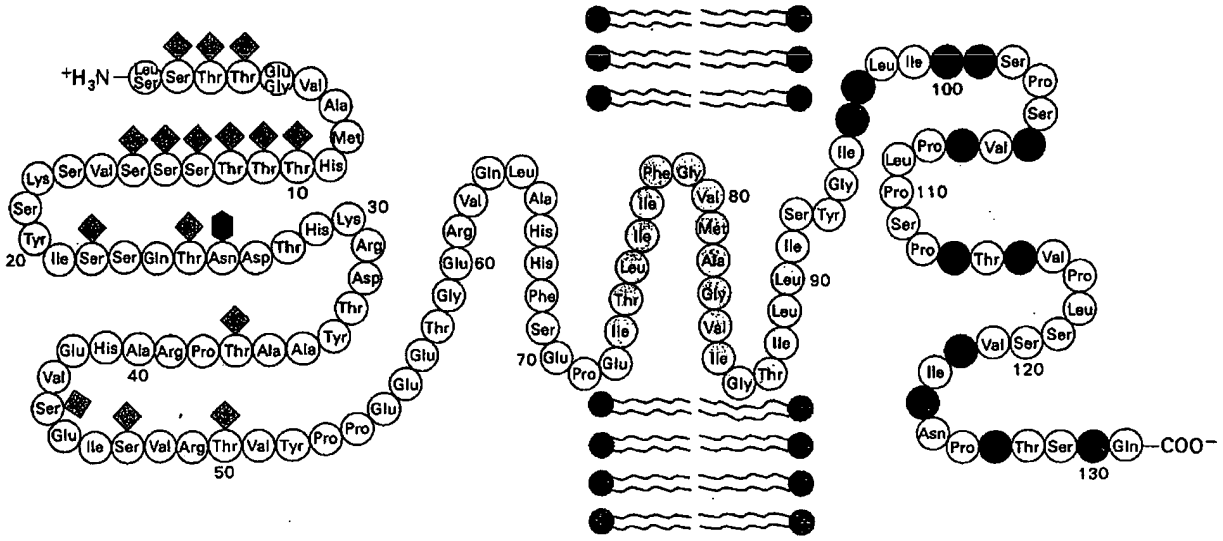
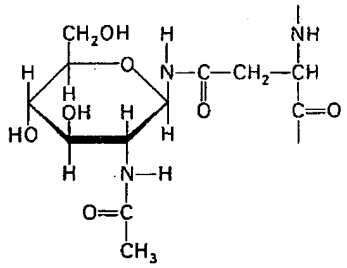


Figure 10-24
Amino acid sequence and transmembrane disposition of glycophorin A from the red-cell membrane. The fifteen carbohydrate units that are attached to serine or threonine residues are shown in light green and the one attached to an asparagine side chain in dark green. The hydrophobic residues buried in the bilayer are shown in yellow. The carboxyl-terminal part of the molecule, located inside the cell, is rich in negatively charged (red) and positively charged (blue) residues. The carbohydrate units in the amino-terminal portion of the molecule outside the cell are rich in negatively charged sialic acid groups. [Courtesy of Dr. Vincent Marchesi.]

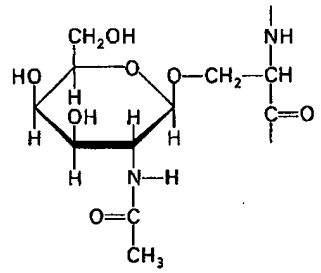
brane; (2) a hydrophobic middle region, which is buried within the hydrocarbon core of the membrane; and (3) a carboxyl-terminal region rich in polar and ionized side chains, which is exposed on the inner face of the red-cell membrane (Figure 10-24). Although much is known about the structure of glycophorin, its function is still an enigma.

CARBOHYDRATE UNITS ARE LOCATED ON THE EXTRACELLULAR SIDE OF PLASMA MEMBRANES

Membranes of eucaryotic cells are usually between 2% and 10% carbohydrate, in the form of *glycolipids* and *glycoproteins*. As mentioned earlier (p. 210), the glycolipids of higher organisms are derivatives of sphingosine with one or more sugar residues. In membrane glycoproteins, one or more chains of sugar residues are attached to serine, threonine, or asparagine side chains of the protein, usually through *N*-acetylglucosamine or *N*-acetylgalactosamine. The location of these carbohydrate groups in membranes can be determined by specific labeling techniques. *Lectins,* which are plant proteins with high affinity for specific sugar residues, are valuable probes in this regard. For example, *concanavalin A* binds to internal and nonreducing terminal α-mannosyl residues, whereas *wheat-germ agglutinin*



***N*-Acetylglucosamine linked to an asparagine residue**



***N*-Acetylgalactosamine linked to a serine residue**

binds to terminal *N*-acetylglucosamine residues. These lectins can be readily seen in electron micrographs if they are conjugated to ferritin, a protein with a very electron dense core of iron hydroxide. The ferritin conjugate of concanavalin A binds specifically to the outer surface of the erythrocyte membrane and not to the inner cytoplasmic surface. The same asymmetry has been observed in the binding of a wide variety of lectins to many kinds of cell membranes. Thus, the localization of the carbohydrate units of the anion channel and glycophorin (Figures 10-23 and 10-24) exemplifies a fundamental theme of membrane structure: *sugar residues are always located on the external surface of plasma membranes. Indeed, the sugar residues in the plasma membranes of all mammalian cells studied thus far are located exclusively on the external surface* (Figure 10-25).

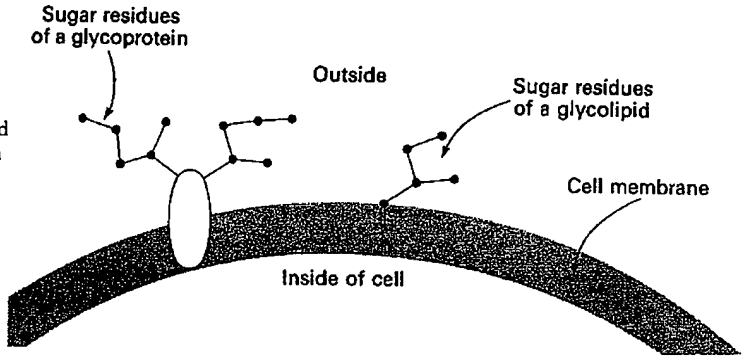


Figure 10-25
Sugar residues of glycoproteins and glycoplipids are usually located on the outside surface of mammalian plasma membranes.

One possible role of carbohydrate groups is to orient glycoproteins in membranes. Because sugars are highly hydrophilic, the sugar residues of a glycoprotein or of a glycolipid will tend to be located at a membrane surface, rather than in the hydrocarbon core. The cost in free energy of inserting an oligosaccharide chain into the hydrocarbon core of a membrane is very high. Consequently, there is a high barrier to the rotation of a glycoprotein from one side of a membrane to the other. The carbohydrate moieties of membrane glycoproteins help to maintain the asymmetric character of biological membranes.

Carbohydrates on cell surfaces may also be important in *intercellular recognition*. The interaction of different cells to form a tissue and the detection of foreign cells by the immune system of a higher organism are examples of processes that depend on the recognition of one cell surface by another. Carbohydrates have the potential for great structural diversity. An enormous number of patterns of surface sugars is possible because (1) monosaccharides can be joined to each other through any of several hydroxyl groups, (2) the C-1 linkage can have either an α or a β configuration, and (3) extensive branching is possible. Indeed, many more different oligosaccharides can be formed from four sugars than oligopeptides from four amino acids.

LIPIDS AND MANY MEMBRANE PROTEINS DIFFUSE RAPIDLY IN THE PLANE OF THE MEMBRANE

Biological membranes are not rigid structures. On the contrary, lipids and many membrane proteins are constantly in lateral motion. The rapid movement of membrane proteins has been visualized by means of fluorescence microscopy. Human cells and mouse cells in culture can be induced to fuse with each other. The resulting hybrid cell is called a *heterokaryon*. Part of the plasma membrane of this heterokaryon comes from a mouse cell, the rest from a human cell. Do the membrane proteins derived from the mouse and human cells stay segregated in the heterokaryon or do they intermingle? This question was answered by using fluorescent-labeled antibodies as markers that could be followed by light microscopy. An antibody specific for mouse membrane proteins was labeled to show a green fluorescence, and an antibody specific for human membrane proteins was labeled to show a red fluorescence (Figure 10-26). In a newly formed heterokaryon, half of the surface displayed green fluorescing patches, the other half red. However, in less than an hour (at 37°C), the red and green fluorescing patches became completely intermixed. This experiment revealed that a *membrane protein can diffuse through a distance of several microns in approximately one minute.*

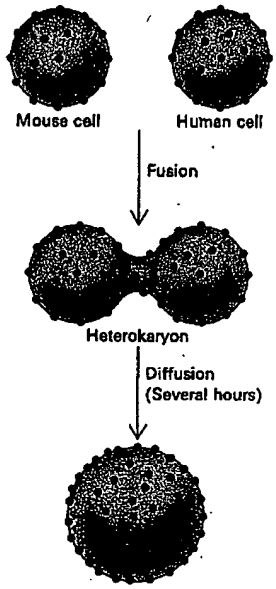


Figure 10-26
Diagram showing the fusion of a mouse cell and a human cell, followed by diffusion of membrane components in the plane of the plasma membrane. The green and red fluorescing markers are completely intermingled after several hours.

A more general and quantitative method for measuring the lateral mobility of membrane molecules in intact cells is the *fluorescence photobleaching recovery technique* (Figure 10-27). First, a cell-surface component is specifically labeled with a fluorescent chromophore. A small region of the cell surface ($\sim 3\ \mu m^2$) is viewed through a fluorescent microscope. The fluorescent molecules in this region are then destroyed by a very intense light pulse from a laser. The fluorescence of this region is subsequently monitored as a function of time using a light level sufficiently low to prevent further bleaching. If the labeled component is mobile, bleached molecules leave and unbleached molecules enter the illuminated region, which results

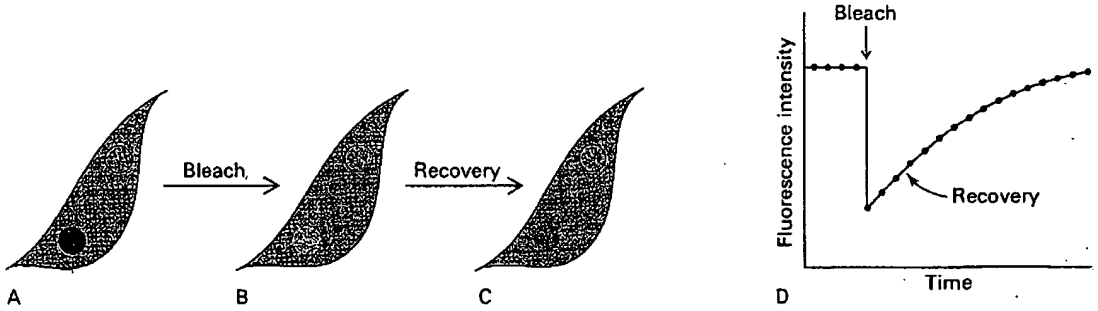


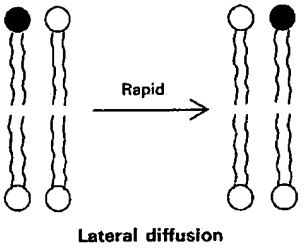
Figure 10-27
Fluorescence photobleaching recovery technique: (A) fluorescence from a labeled cell-surface component in a small illuminated region of a cell; (B) the fluorescent molecules are bleached by an intense light pulse; (C) the fluorescence intensity recovers as bleached molecules diffuse out and unbleached molecules diffuse into the illuminated region; (D) the rate of recovery depends on the diffusion coefficient.

in an increase in the fluorescence intensity. The rate of recovery of the fluorescence level depends on the lateral mobility of the fluorescent-labeled component, which can be expressed in terms of a diffusion coefficient D. The average distance s (in cm) traversed in two dimensions in time t (sec) depends on D ($cm^2\ sec^{-1}$) according to

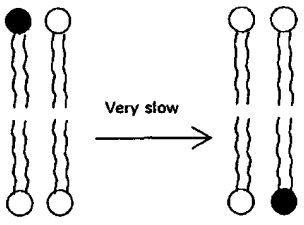
$$s = (4\,Dt)^{1/2}$$

The diffusion coefficient of lipids in a variety of membranes is about $10^{-8}\ cm^2\ sec^{-1}$. Thus, a phospholipid molecule diffuses an average distance of 2×10^{-4} cm, or 2 μm, in 1 second. This means that a *lipid molecule can travel from one end of a bacterium to the other in a second.* The magnitude of the observed diffusion coefficient indicates that the viscosity of the membrane is about one hundred times that of water, rather like that of olive oil.

In contrast, proteins vary markedly in their lateral mobility. *Some proteins are nearly as mobile as lipids, whereas others are virtually immobile.* For example, the photoreceptor protein rhodopsin, a highly mobile protein, has a diffusion coefficient of $4 \times 10^{-9}\ cm^2\ sec^{-1}$. At the other extreme is *fibronectin,* a peripheral glycoprotein that participates in cell-substratum interactions, for which D is less than $10^{-12}\ cm^2\ sec^{-1}$. Some of the less-mobile proteins may be anchored to submembranous cytoplasmic filaments.



Lateral diffusion



Transverse diffusion
(Flip-flop)

MEMBRANE PROTEINS DO NOT ROTATE ACROSS BILAYERS

The spontaneous rotation of lipids from one side of a membrane to the other is a very slow process, in contrast with their movement parallel to the plane of the bilayer. The transition of a molecule from one membrane surface to the other is called *transverse diffusion,* or *flip-flop,* whereas diffusion in the plane of a membrane is termed *lateral diffusion.* The flip-flop of phospholipid molecules in phosphatidyl choline vesicles has been directly measured by electron spin resonance techniques, which showed that *a phospholipid molecule flip-flops once in several hours* (see problem 5, p. 231, for the experimental design). Thus, a phospholipid molecule takes about 10^9 times as long to flip-flop across a 50-Å membrane as it takes to diffuse a distance of 50 Å in the lateral direction.

The free-energy barriers to the flip-flopping of protein molecules are even larger than for lipids because proteins have more extensive polar regions. In fact, *the flip-flop of a protein molecule has not been observed.* Hence, *membrane asymmetry can be preserved for long periods.*

FLUID MOSAIC MODEL OF BIOLOGICAL MEMBRANES

In 1972, S. Jonathan Singer and Garth Nicolson proposed a fluid mosaic model for the gross organization of biological membranes. The essence of their model is that membranes are *two-dimensional*

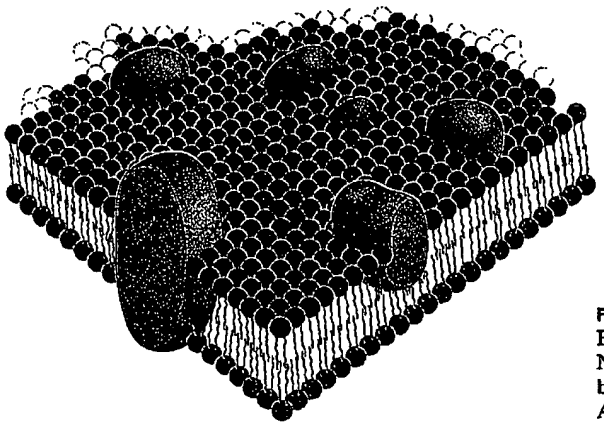


Figure 10-28
Fluid mosaic model. [After S. J. Singer and G. L. Nicolson. *Science* 175(1972):723. Copyright 1972 by the American Association for the Advancement of Science.]

solutions of oriented globular proteins and lipids (Figure 10-28). This proposal is supported by a wide variety of experimental observations. The major features of this model are:

1. Most of the membrane phospholipid and glycolipid molecules are in bilayer form. This lipid bilayer has a dual role: it is both a *solvent* for integral membrane proteins and a *permeability barrier.*

2. A small proportion of membrane lipids interact specifically with particular membrane proteins and may be essential for their function.

3. Membrane proteins are free to diffuse laterally in the lipid matrix unless restricted by special interactions, whereas they are not free to rotate from one side of a membrane to the other.

MEMBRANES ARE ASYMMETRIC

Membranes are structurally and functionally asymmetric, as exemplified by the orientations of glycophorin and the anion channel and, more generally, by the external localization of membrane carbohydrates. The outer and inner surfaces of *all known biological membranes have different components and different enzymatic activities.* A clear-cut example is provided by the pump that regulates the concentration of Na^+ and K^+ ions in cells. This transport system is located in the plasma membrane of nearly all cells in higher organisms. The Na^+-K^+ pump assembly is oriented in the plasma membrane so that it pumps Na^+ out of the cell and K^+ into it (Figure 10-29). Furthermore, ATP must be on the inside of the cell to drive the pump. Ouabain, a specific inhibitor of the pump, is effective only if it is located outside.

As will be discussed in detail in a later chapter (p. 718), membrane proteins have a unique orientation because they are synthesized and inserted in an asymmetric manner. This absolute asym-

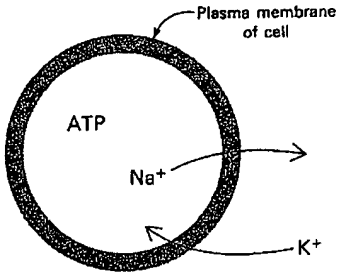


Figure 10-29
Asymmetry of the Na^+-K^+ transport system in plasma membranes.

metry is preserved by the absence of transmembrane movement of these proteins throughout their lifetime in the membrane. Lipids, too, are asymmetrically distributed as a consequence of their mode of biosynthesis, but this asymmetry is usually not absolute, except in the case of glycolipids. For example, in the red-cell membrane, sphingomyelin and phosphatidylcholine are preferentially located in the outer leaflet of the bilayer, whereas phosphatidyl ethanolamine and phosphatidyl serine are mainly in the inner leaflet. Large amounts of cholesterol are present in both leaflets. The functional significance of lipid asymmetry is not yet understood.

MEMBRANE FLUIDITY IS CONTROLLED BY FATTY ACID COMPOSITION AND CHOLESTEROL CONTENT

The fatty acyl chains of lipid molecules in bilayer membranes can exist in an ordered, rigid state or in a relatively disordered, fluid state. In the ordered state, all of the C—C bonds have a *trans* conformation, whereas in the disordered state, some are in the *gauche* conformation (Figure 10-30).

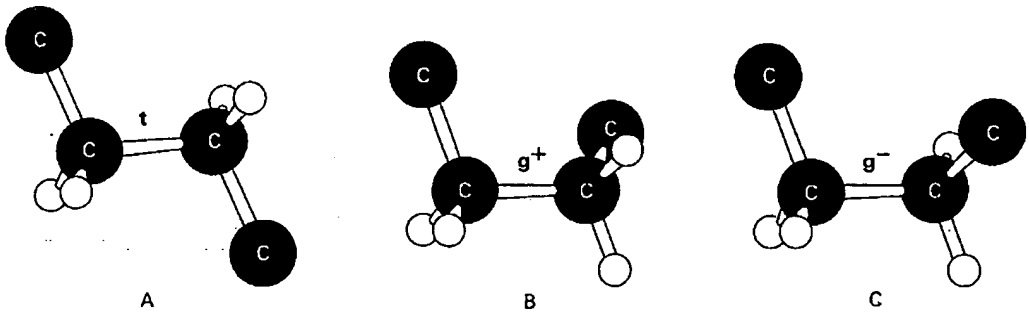


Figure 10-30
Conformation of C—C bonds in fatty acyl chains: (A) *trans* (t) conformation; (B and C) a 120-degree rotation yields a *gauche* (g) conformation, which can be either g^+ (clockwise rotation) or g^- (counterclockwise rotation). A gauche conformation bends a hydrocarbon chain by 120 degrees.

The transition from the rigid (all *trans*) to the fluid (partially *gauche*) state occurs as the temperature is raised above T_m, the melting temperature. *This transition temperature depends on the length of the fatty acyl chains and on their degree of unsaturation.* The rigid state is favored by the presence of saturated fatty acyl residues because their straight hydrocarbon chains interact very favorably with each other (Figure 10-31A). On the other hand, *a* cis *double bond produces a bend in the hydrocarbon chain. This bend interferes with a highly ordered packing of fatty acyl chains* and so T_m is lowered (Figure 10-31B). The length of the fatty acyl chain also affects the transition temperature. Long hydrocarbon chains interact more strongly than do short ones. Specifically, each additional $—CH_2—$ group makes a favorable contribution of about -0.5 kcal/mol to the free energy of interaction of two adjacent hydrocarbon chains.

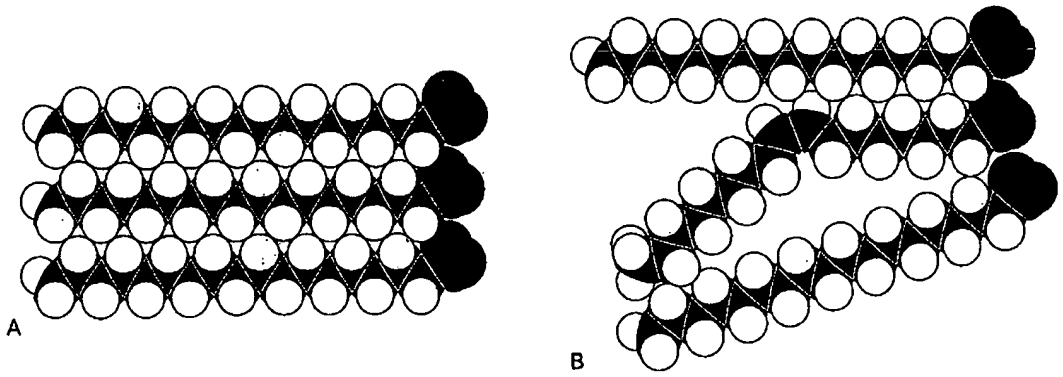


Figure 10-31
The highly ordered packing of fatty acid chains is disrupted by the presence of *cis* double bonds. These space-filling models show the packing of (A) three molecules of stearate (C_{18}, saturated) and (B) a molecule of oleate (C_{18}, unsaturated) between two molecules of stearate.

Procaryotes regulate the fluidity of their membranes by varying the number of double bonds and the length of their fatty acyl chains. For example, the ratio of saturated to unsaturated fatty acyl chains in the *E. coli* membrane decreases from 1.6 to 1.0 as the growth temperature is lowered from 42° to 27°C. This decrease in the proportion of saturated residues prevents the membrane from becoming too rigid at the lower temperature. *In eucaryotes, cholesterol also is a key regulator of membrane fluidity.* Cholesterol prevents the crystallization of fatty acyl chains by fitting between them. In fact, cholesterol abolishes the phase transition. An opposite effect of cholesterol is to sterically block large motions of fatty acyl chains and thereby make the membrane less fluid. Thus, cholesterol moderates the fluidity of membranes.

THREE-DIMENSIONAL IMAGES OF MEMBRANES CAN BE RECONSTRUCTED FROM ELECTRON MICROGRAPHS

The power of x-ray crystallography in elucidating the three-dimensional structure of soluble proteins is clearly established, as exemplified by the oxygen carriers and enzymes that were discussed in previous chapters. What about x-ray crystallographic analyses of membrane proteins? The difficulty is that three-dimensional crystals of integral membrane proteins have not yet been obtained. However, some membrane proteins give rise to ordered lattices in the plane of the membrane—in other words, they form two-dimensional crystals. These crystalline sheets are suitable for structural analyses by electron microscopy, as shown for the *purple membrane* of *Halobacterium halobium*, a salt-loving bacterium. This specialized region of the cell membrane contains *bacteriorhodopsin*, a 25-kdal protein, which converts light into a transmembrane proton gradient that is used to synthesize ATP (p. 451). Crystalline sheets with

diameters as large as 1 μm can be obtained. The significance of having about 20,000 bacteriorhodopsin molecules in this crystalline array is that an image can be obtained with a very low dose of electrons and so there is little radiation damage. Furthermore, unstained samples can be analyzed to obtain a high-resolution image. A single electron micrograph of a crystalline sheet of the purple membrane yields a view of the structure projected onto a plane. The next stage is to tilt the sample and recombine the information from some twenty electron micrographs by Fourier techniques.

Using this technique, Richard Henderson and Nigel Unwin reconstructed a three-dimensional image of the purple membrane at 7-Å resolution (Figure 10-32). *The protein in this membrane contains seven closely packed α helices, which extend nearly perpendicular to the plane of the membrane for most of its 45-Å width.* Lipid bilayer regions fill the spaces between the protein molecules. The purple membrane probably exemplifies a structural motif that will be found in other integral membrane proteins. In particular, it seems likely that other membrane pumps and channels will have α-helical segments spanning the bilayer.

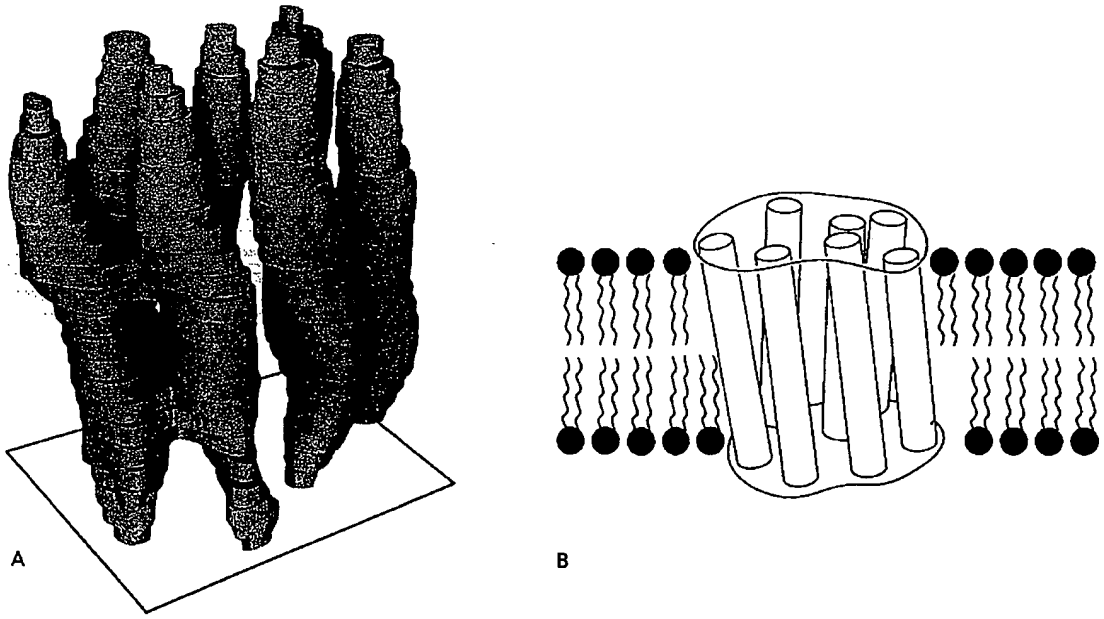


Figure 10-32
A. Model of bacteriorhodopsin constructed from a 7-Å three-dimensional map. B. Interpretative diagram showing the arrangement of α-helical segments in the lipid bilayer. The connections between these helices are not yet known. [Courtesy of Dr. Richard Henderson and Dr. Nigel Unwin.]

SUMMARY

Biological membranes are sheetlike structures, typically 75 Å wide, that are composed of protein and lipid molecules held together by noncovalent interactions. Membranes are highly selective permeability barriers. They create closed compartments, which may be an entire cell or an organelle within a cell. Pumps and gates in membranes regulate the molecular and ionic compositions of these compartments. Membranes also control the flow of information between cells. For example, some membranes contain receptors for hormones such as insulin. Furthermore, membranes are intimately involved in such energy conversion processes as photosynthesis and oxidative phosphorylation.

The major classes of membrane lipids are phospholipids, glycolipids, and cholesterol. Phosphoglycerides, a type of phospholipid, consist of a glycerol backbone, two fatty acid chains, and a phosphorylated alcohol. The fatty acid chains usually contain between 14 and 24 carbon atoms; they may be saturated or unsaturated. Phosphatidyl choline, phosphatidyl serine, and phosphatidyl ethanolamine are major phosphoglycerides. Sphingomyelin, a different type of phospholipid, contains a sphingosine backbone instead of glycerol. Glycolipids are sugar-containing lipids derived from sphingosine. A common feature of these membrane lipids is that they are amphipathic molecules. They spontaneously form extensive bimolecular sheets in aqueous solutions because they contain both a hydrophilic and a hydrophobic moiety. These lipid bilayers are highly impermeable to ions and most polar molecules, yet they are quite fluid, which enables them to act as a solvent for membrane proteins.

Distinctive membrane functions such as transport, communication, and energy transduction are mediated by specific proteins. Some membrane proteins are deeply imbedded in the hydrocarbon region of the lipid bilayer. Transmembrane proteins such as the band-3 protein from erythrocytes can serve as ion channels. Membranes are structurally and functionally asymmetric, as exemplified by the directionality of ion transport systems in them and the localization of sugar residues on the external surface of mammalian plasma membranes. Membranes are dynamic structures in which proteins and lipids diffuse rapidly in the plane of the membrane (lateral diffusion), unless restricted by special interactions. In contrast, the rotation of proteins and lipids from one side of a membrane to the other (transverse diffusion, or flip-flop) is usually very slow. The degree of fluidity of membranes partly depends on the chain length and the extent to which their constituent fatty acids are unsaturated.

SELECTED READINGS

WHERE TO START

Singer, S. J., and Nicolson, G. L., 1972. The fluid mosaic model of the structure of cell membranes. *Science* 175:720–731.

Raff, M. C., 1976. Cell-surface immunology. *Sci. Amer.* 234(5):30–39. [A lucid and well-illustrated article showing how antibodies have contributed to our understanding of cell-surface structure and dynamics. Available as *Sci. Amer.* Offprint 1338.]

Rothman, J. E., and Lenard, J., 1977. Membrane asymmetry. *Science* 195:743–753.

BOOKS AND REVIEWS

Bretscher, M. S., and Raff, M. C., 1975. Mammalian plasma membranes. *Nature* 258:43–49.

Bretscher, M. S., 1973. Membrane structure: some general principles. *Science* 181:622–629.

Ashwell, G., and Morell, A. G., 1977. Membrane glycoproteins and recognition phenomena. *Trends Biochem. Sci.* 2:76–78.

Tyrrell, D. A., Heath, T. D., Colley, C. M., and Ryman, B. E., 1976. New aspects of liposomes. *Biochim. Biophys. Acta* 457:259–302.

Poste, G., and Nicolson, G. L., (eds.), 1977. *Dynamic Aspects of Cell Surface Organization.* North-Holland. [Contains articles on membrane dynamics, cell-surface immunology, and changes in cell-surface proteins during cell growth and malignant transformation.]

Tanford, C., 1980. *The Hydrophobic Effect: Formation of Micelles and Biological Membranes,* 2nd ed. Wiley-Interscience. [A lucid statement of thermodynamic aspects of membrane structure.]

MEMBRANE DYNAMICS

Frye, C. D., and Edidin, M., 1970. The rapid intermixing of cell surface antigens after formation of mouse-human heterokaryons. *J. Cell Sci.* 7:319–335.

Kornberg, R. D., and McConnell, H. M., 1971. Inside-outside transitions of phospholipids in vesicle membranes. *Biochemistry* 10:1111–1120.

Cone, R. A., 1972. Rotational diffusion of rhodopsin in the visual receptor membrane. *Nature New Biol.* 236:39–43.

Poo, M., and Cone, R. A., 1974. Lateral diffusion of rhodopsin in the photoreceptor membrane. *Nature* 247:438–441.

Elson, E. L., and Schlessinger, J., 1979. Long-range motions on cell surfaces. *In* Schmitt, F. O., and Worden, F. G., (eds.), *The Neurosciences: Fourth Study Program,* pp. 691–701. MIT Press. [Discussion of the fluorescence photobleaching recovery technique and its use in measuring the lateral diffusion of cell-surface components.]

Seelig, A., and Seelig, J., 1977. Effect of a single *cis* double bond on the structure of a phospholipid bilayer. *Biochemistry* 16:45–50.

ELECTRON MICROSCOPY OF MEMBRANES

Branton, D., 1966. Fracture faces of frozen membranes. *Proc. Nat. Acad. Sci.* 55:1048–1056.

Henderson, R., and Unwin, P. N. T., 1975. Three-dimensional model of purple membrane obtained by electron microscopy. *Nature* 257:28–32. [The first view of a membrane protein. At 7-Å resolution, transmembrane helices are evident.]

Unwin, P. N. T., and Henderson, R., 1975. Molecular structure determination by electron microscopy of unstained crystalline specimens. *J. Mol. Biol.* 94:425–440. [Presentation of the experimental strategy for determining the structure of membrane proteins that form highly ordered two-dimensional arrays.]

RED-CELL MEMBRANE

Steck, T. L., 1974. The organization of proteins in the human red blood cell membrane. *J. Cell Biol.* 62:1–19.

Marchesi, V. T., Furthmayr, H., and Tomita, M., 1976. The red cell membrane. *Ann. Rev. Biochem.* 45:667–698.

Steck, T. L., 1978. The band 3 protein of the human red cell membrane: a review. *J. Supramol. Struct.* 8:311–324.

SYNTHETIC AND RECONSTITUTED MEMBRANES

Fleischer, S., and Packer, L., (eds.), 1974. *Methods in Enzymology,* vol. 32. Academic Press. [Contains excellent articles on experimental methods in membrane research, including procedures for the preparation of model and reconstituted membranes.]

Darszon, A., Vandenberg, E. A., Ellisman, M. H., and Montal, M., 1979. Incorporation of membrane proteins into large single bilayer vesicles. *J. Cell Biol.* 81:446–452.

Racker, E., 1977. Perspectives and limitations of resolutions-reconstitution experiments. *J. Supramol. Struct.* 6:215–228.